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Quantitative Determination of the Binding Capacity of the Sex Hormone-Binding Globulin, Using Agar Gel Electrophoresis

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Summary: An easy, rapid and precise technique for the determination of the binding capacity of the sex hormone-binding globulin (SHBG) is described. The plasma is incubated with [^{3}H]5 α-dihydrotestosterone (DHT) either alone, or together with an excess of unlabelled DHT. The SHBG-binding peaks are demonstrated by agar gel electrophoresis according to *Wagner* ((1972) Hoppe-Seylers Z. Physiol. Chem. *353*, 1235–1245), and the SHBG-binding capacity is calculated from the radioactivity specifically bound to the SHBG peak, corrected for the dissociation of the [^{3}H]DHT from SHBG during the 90 minute electrophoresis. In addition to investigating various problems of methodology, the following clinical results were obtained: 1. Normal range ($\bar{x} \pm s$) of the SHBG-binding capacity for males (age 22–41; n = 26) 38 ± 11 nmol/l. 2. An older male group has, with 48 ± 10 nmol/l, a significantly higher binding capacity. 3. The highest values found so far were in pregnancy plasma of the third trimenon (330 and 400 nmol/l).

Quantitative Bestimmung der Bindungskapazität des Sexualhormon-bindenden Globulins mittels Agargelelektrophorese

Zusammenfassung: Beschrieben wird eine einfache, schnelle und präzise Methode zur Bestimmung der Bindungskapazität des Sexualhormon-bindenden Globulins (SHBG). Die Hauptschritte beinhalten die Inkubation des Plasma mit [^{3}H]5α-Dihydrotestosteron (DHT) allein oder zusammen mit einem gegenüber dem tritiierten DHT im Überschuß zugegebenen unmarkierten DHT, die Darstellung des SHBG-Peaks mittels Agargelelektrophorese nach *Wagner*, sowie die Errechnung der Bindungskapazität des SHBG aufgrund der spezifisch im SHBG-Peak gebundenen Radioaktivität unter Berücksichtigung der Dissoziation von [^{3}H]DHT und SHBG während der 90-minütigen Elektrophorese. Neben Antworten auf methodische Fragen werden drei klinisch relevante Befunde mitgeteilt: 1. Der Normalbereich ($\bar{x} \pm s$) der SHBG-Bindungskapazität für Männer (Altersbereich 22–41 Jahre; n = 26) beträgt 38 ± 11 nmol/l. Eine ältere männliche Probandengruppe hat mit 48 ± 10 nmol/l eine signifikant höhere SHBG-Bindungskapazität. 3. Bisher wurden die höchsten Werte (330 und 400 nmol/l) im Plasma Schwangerer des dritten Trimenon gefunden.

Introduction

In 1966 *Mercier* et al. (1) described a plasma protein which binds testosterone with high affinity. In the same year *Rosenbaum* et al. (2) found a plasma protein displaying high affinity for estradiol-17β. Later, the common identity of both binding proteins was clearly shown by *DeMoor* et al. (3). In order to understand the physiological role of this sex hormone-binding globulin (SHBG), its quantitative determination is necessary. Most of the respective techniques are based on the same principle:

(a) saturation of the binding sites by tritiated steroids,
(b) followed by separation of the SHBG-bound radioactivity from unspecifically bound and free radioactivity,
(c) calculation of the SHBG-binding capacity by the amount of SHBG-bound tritiated steroid.

Various procedures have been proposed for the separation of the steroid-SHBG complex, as reviewed very recently by *Lutz* et al. (4): classical equilibrium dialysis, microdialysis with Sephadex G-25, conventional gel filtration, competitive absorption using dextran-coated charcoal or florisil, and precipitation with ammonium sulphate. Less frequently used in this respect are the polyacrylamide gel electrophoresis (5, 6), DAEA-cellulose filter paper (7) and equilibrium partition in an aqueous two-phase system (8).

For several years we have used agar gel electrophoresis according to *Wagner* (9) for the determination of androgen receptors in target tissues (10, 11). In an abstract, *Wagner & Rüffert* (12) first reported that agar

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gel electrophoresis can also be used for quantifying the SHBG-binding capacity. This technique allows an easy, rapid and precise demonstration of the SHBG-[^{3}H]5α-dihydrotestosterone (DHT) complex, the method therefore being highly suitable as a routine assay for the determination of the SHBG-binding capacity.

The detailed description and evaluation of the technique, the comparison of the data with values obtained by a SHBG-binding capacity determination according to *Dennis* et al. (10), and the assessment of a normal range of the SHBG-binding capacity in male plasma are the subjects of the report.

Material and Methods

Heparinized blood was obtained from normal male blood donors. It was immediately centrifuged (20 min at 3000 rpm) and the plasma stored at –18 °C.

Chemicals and solutions

[1, 2-^{3}H]5α-Dihydrotestosterone (DHT; specific activity 1628 TBq/mol (44 Ci/mmol); purity 96%) was obtained from NEN Chemicals (Dreieichenhain). The radioactive steroid in benzene/ethanol (9 + 1, volumes) was evaporated to dryness and the residue was redissolved in physiological NaCl solution/ethanol (9 + 1, volumes) to obtain a solution of 57.7 MBq/l (1.56 mCi/l) = 37 nmol/l. Unlabelled DHT and all other reagents, unless otherwise stated, were obtained from Merck AG (Darmstadt). A 37 μmol/l solution of DHT was prepared in physiological NaCl solution/ethanol (9 + 1, volumes).

Electrophoresis buffer: Na-diethylbarbiturate-acetate-HCl, pH 8.2 at 0 °C, ionic strength $\mu = 0.1$.

Charcoal suspension: 260 g/l Norit A (Serva, Heidelberg), 2 g/l Dextran T70 (Pharmacia, Uppsala) in Tris-HCl (0.01 mol/l, pH 7.4 at 20 °C).

Scintillation fluid: 240 g naphthalene, 15 g 2,2'-*p*-phenylenebis-(5-phenyloxazole), 0.15 g 2,5-diphenyloxazole, made up to 3 litres with dioxan/xylene (2 1 + 1 1).

Incubation

The procedures were carried out at 4 °C. Plasma was diluted 1 : 21 with Tris-HCl-buffer. Tritiated DHT was added, giving a final [^{3}H]DHT concentration of 5.2 nmol/l. In a parallel test tube tritiated DHT together with unlabelled DHT were added to plasma, giving a final concentration of 5.2 nmol/l for [^{3}H]DHT and 5.2 μmol/l for DHT. Incubation period: 14 hours at 0 °C.

In preliminary experiments, plasma was treated with charcoal before the steroid incubation: 0.2 ml of the charcoal suspension was added to 0.22 ml of diluted plasma (diluted 1 : 11 with Tris-HCl buffer). The mixture was stirred overnight at 4 °C. After this period the dextran-coated charcoal was removed by centrifugation at 5 000 rpm for 4 minutes, and the supernatant used for further incubation steps.

Agar gel electrophoresis

This technique was inaugurated by *Wagner* (9). The apparatus for punching and slicing the gel layer was purchased from the workshop of the Max-Planck-Institut für Zellbiologie (Wilhelmshaven). Preparation of agar/agarose gels: 1.35 g pure agar (Behringwerke, Marburg) and 0.15 g agarose (Serva, Heidelberg) were made up to 75 ml H_2O plus 75 ml electrophoresis buffer, boiled briefly and allowed to gel in a Plexiglas mould. Two gel layers of 95 × 100 × 5 mm were cut out and slid onto 95 × 95 × 0.6 mm glass plates, one end of the gel overlapping the plate by 5 mm. The gels can be stored at 4 °C in a humid chamber for several days. For the application of plasma samples (40 μl per hole), 10 holes are punched at right angles to the direction of separation in the middle of the gel layer, between the anodic and cathodic end.

The glass plates, bearing the gel layers, are placed on a teflon-coated metal block (Hübscher, Hamburg), which is cooled at –2 °C by a cryostat K4R (Meßgerätewerk, Lauda). Attachment of the ends of the gels to anode and cathode respectively is by means of filter paper bridges (140 × 100 mm), dipping into the electrophoresis buffer. The bridges consist at the cathode of two layers of filter paper MN 866 (Macherey & Nagel, Düren), at the anode of one filter paper MN 604, underlying the gel end which overlaps the glass plate, followed by one MN 866 and one MN 604 filter paper, the latter covering the gel end by approximately 5 mm. After filling the sample holes with plasma, the electrophoresis (power supply type 121260, Desaga, Heidelberg) was run 90 minutes at 10 Volt/cm (circa 130 mA, 300 V), the temperature in the gel being + 4 °C. The metal block and buffer trays are covered by a perspex lid (Hübscher, Hamburg). After electrophoresis, the gel was cut into 10 strips parallel to the direction of separation with a sample-hole in the middle of each strip. The strips were finally divided into 3 mm wide gel slices at right angles to the direction of separation.

Measurement of radioactivity

The 3 mm wide gel slices were deep frozen for 2 hours at – 18 °C in counting vials in order to facilitate elution of the radioactivity by the scintillation fluid. After addition of the scintillation fluid, the counting vials were shaken for 1–2 hour and then analyzed in a scintillation counter (Packard TriCarb 3390, efficiency 32%). Because of the constant ratio to the internal standard, the radioactivity was measured in counts/min.

Calculation of the SHBG-binding capacity

Under the assumption that SHBG is completely saturated by [^{3}H]DHT, the amount of bound [^{3}H]DHT is equal the SHBG-binding capacity. Furthermore, if a mol to mol binding relationship between steroid and SHBG is assumed (13, 14), the concentration of SHBG-bound hormone is equal to the SHBG concentration. The SHBG-binding capacity M is calculated by the equation: $M = A \times F + K$, where A = activity (counts/min), estimated by subtracting from the totally bound [^{3}H]DHT in the SHBG peak the background; F = factor, which corrects for efficiency of the scintillation counter, for specific activity of the [^{3}H]DHT, and for plasma dilution; K = factor, which corrects for the dissociation of [^{3}H]DHT from the SHBG during the run. If the SHBG-bound [^{3}H]DHT decreases by 28.5% per 90 minute the corresponding K is 40%. (Example: During the run the peak of 1 000 counts/min decreases by 28.5% to 715 counts/min, i.e. if after a 90 minute run 715 cpm are found, then 285 counts/min (i.e. 40% of 715 counts/min) must be added in order to obtain a peak value of 1 000 counts/min).

SHBG-binding capacity determination according to Dennis et al.

This technique has been described in detail elsewhere (15).

Statistics

1. *Spearman* rank correlation was performed when comparing the binding data obtained by the two methods used.
2. The significant differences in SHBG-binding capacity between the younger and older group were checked by *Student*-t-test, assuming a normal distribution of the data.

Results

When incubating human plasma with tritiated DHT alone, or together with a 1000–fold excess of unlabelled DHT, binding patterns as shown in figure 1 were obtained by agar gel electrophoresis. The SHBG-bound radioactivity was found cathodically (right from the start) in slice numbers 15–18, while unbound, e.g. free radioactivity was present in slice numbers 19–28. As outlined in Material and Methods, the binding difference of [^{3}H]DHT in slice numbers 15–18 (shaded area) is taken for the calculation of the SHBG-

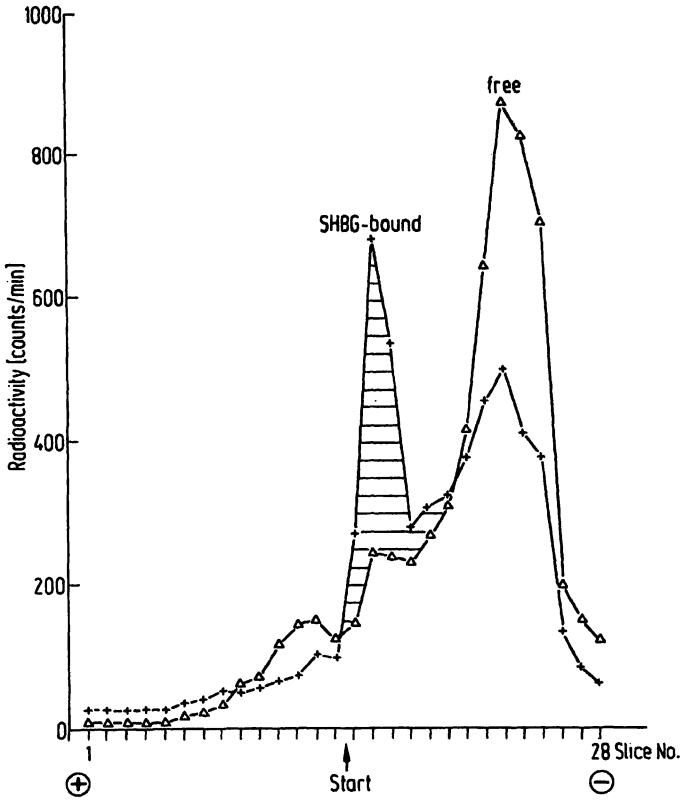

Fig. 1. Binding and displacement of [^{3}H]5-α-dihydrotestosterone (DHT) in male plasma, analyzed by agar gel electrophoresis. Plasma (1:21 diluted with Tris-HCl buffer) was incubated for 14 hours at 0 °C with 5.2 nmol/l [^{3}H]DHT alone (+) or in the presence of a 1000-fold excess (5.2 μmol/l) of unlabelled DHT (Δ). The peak difference in slice numbers 15–18 (shaded area) indicates the SHBG-bound [^{3}H]DHT.

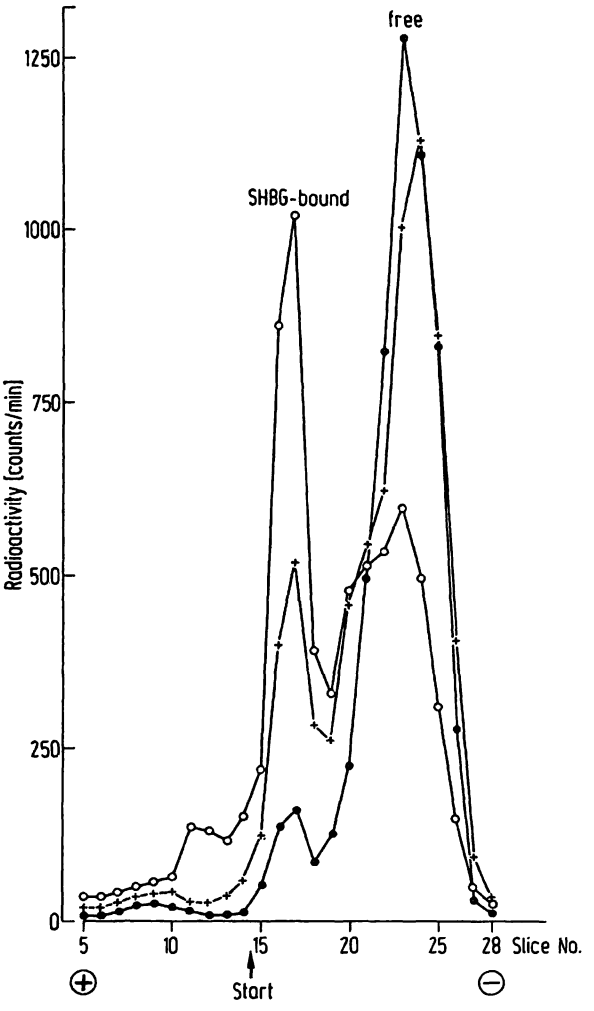

Fig. 2. Binding of [^{3}H]5α-dihydrotestosterone (DHT) in different dilutions of male plasma, analyzed by agar gel electrophoresis. Plasma was diluted 1:11 (○), 1:21 (+) and 1:51 (●) with Tris-HCl buffer and incubated with [^{3}H]DHT in a final concentration of 5.2 nmol/l.

binding capacity. A small quantity of the displaced radioactivity is shifted to the anodic part of the gel, while the majority is recovered in the free fraction.

Figure 2 shows that the SHBG peak decreases with increasing dilution of the plasma, incubated with a final [^{3}H]DHT concentration of 5.2 nmol/l. In figure 3 it is shown that using constant amounts of [^{3}H]DHT and [^{3}H]DHT plus unlabelled DHT respectively, the SHBG-bound radioactivity decreases linearily with the plasma dilution, indicating that the SHBG-binding is saturated under the experimental conditions used. Also we found that the incubation of 1 : 21 diluted male plasma with increasing concentrations of tritiated DHT (5.2 nmol/l – 110 nmol/l) and tritiated DHT plus unlabelled DHT respectively does not increase the SHBG-bound radioactivity. When analyzing 1 : 21 diluted pregnancy plasma (37th week of gestation) saturation of the SHBG-bound radioactivity was obtained when using 52 nmol/l [^{3}H]DHT.

As shown in figure 4, pretreatment of the plasma by an overnight charcoal incubation, in order to remove endogenous steroid from the SHBG, does not lead to a higher binding capacity and was therefore omitted.

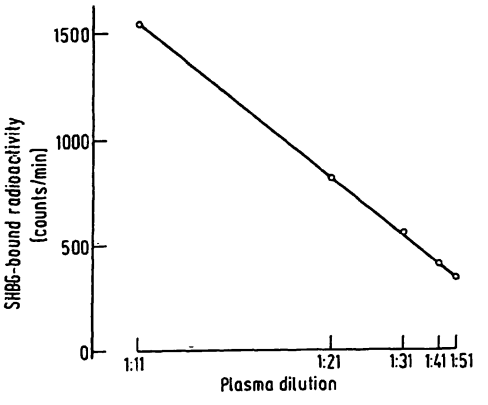

Fig. 3. Relationship between SHBG-bound [^{3}H]5α-dihydrotestosterone (DHT) and plasma dilution. The final concentration of added [^{3}H]DHT was constant (5.2 nmol/l), and the plasma was diluted with Tris-HCl buffer. The binding data were obtained by agar gel electrophoresis. Mean values of three experiments are shown with deviation from experimental values of < 5%.

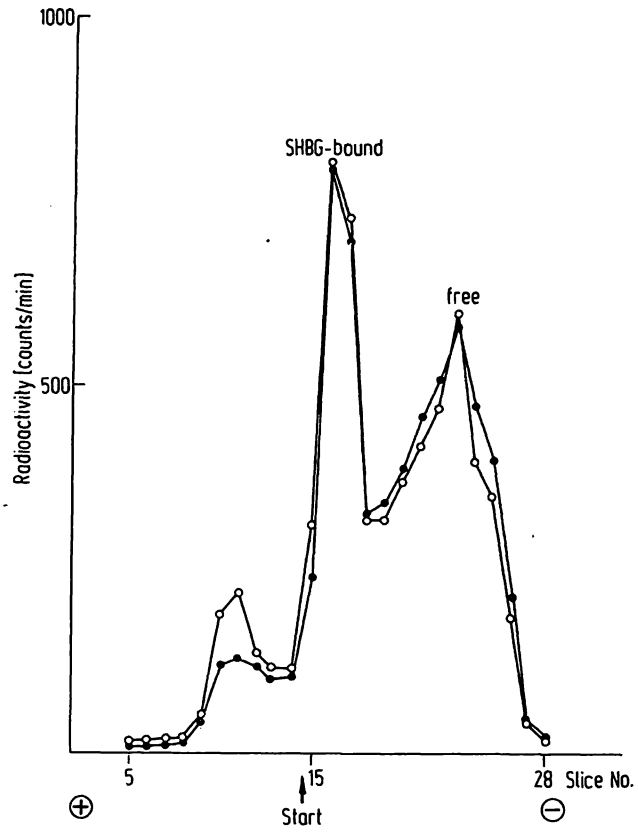

Fig. 4. Binding of [^{3}H]5α-dihydrotestosterone (DHT) to SHBG of plasma, non-treated (•) or treated (○) with charcoal overnight, before [^{3}H]DHT was added (for further details see Material and Methods).

During the electrophoresis, a time-dependent amount of SHBG-bound DHT will be dissociated from SHBG, as shown in figure 5. The linearity of the time-dependent dissociation is plotted in figure 6. Two experiments give nearly identical results, the average rate being 19% per hour. In order to determine the actual SHBG-binding capacity the SHBG-bound DHT after a 90 minute electrophoresis must be corrected for the dissociation rate (see Material and Methods).

The precision of the method was checked by performing 10 determinations of a male plasma sample within one electrophoresis (intra-assay); the coefficient of variation was 3.7%, the inter-assay coefficient of variation being 9.8% (n = 8). Due to the lack of purified SHBG, accuracy could not be determined. An indirect evaluation of the accuracy of the method is shown in figure 7, where 10 male plasma samples were simultaneously analyzed by agar gel electrophoresis and by a method first described by *Dennis* et al. from this laboratory (15).

In figure 8 the normal range ($\bar{x} \pm s$) of the SHBG-binding capacity of 26 blood donors (aged 22—41) is summarized. In table 1 the range is compared with values derived by other techniques.

Plasma of males aged 48—64 shows significantly ($P < 0.02$) higher SHBG-binding capacities (48 ± 10 nmol/l) than plasma of the younger group. The highest SHBG-binding capacities are found in two pregnancy plasmas, the values being 330 and 400 nmol/l respectively.

Discussion

In order to use routinely the method described above as a tool for obtaining relevant values for the plasma SHBG-binding capacity, the SHBG-binding peak must be saturated. Plasma dilution and the amount of [^{3}H] DHT added are the two parameters by which saturation

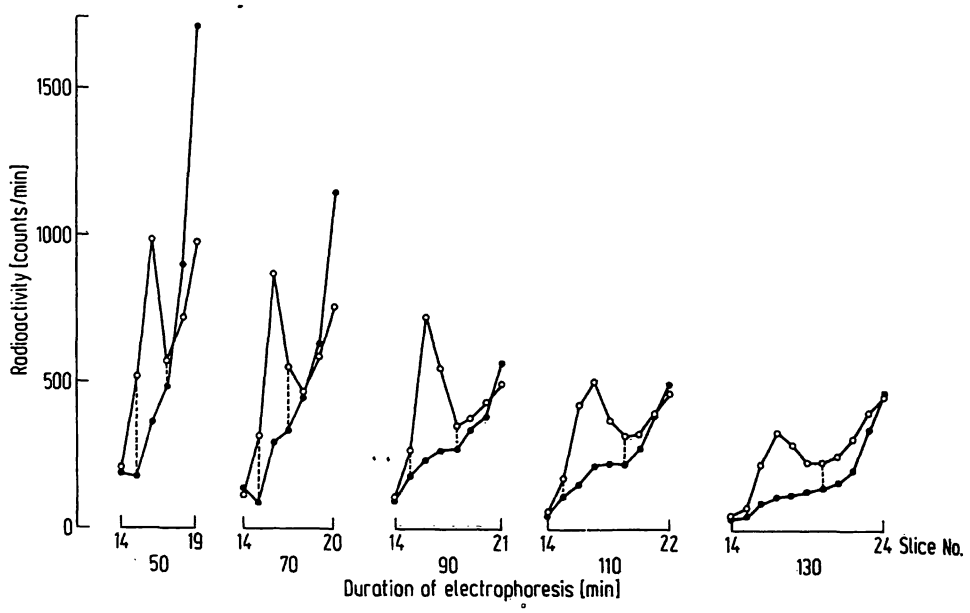

Fig. 5. Dissociation of the SHBG-[^{3}H]5α-dihydrotestosterone (DHT)-binding during agar gel electrophoresis. Male plasma, diluted 1:21 with Tris-HCl buffer was incubated with [^{3}H]DHT alone (○) or with [^{3}H]DHT plus a 1000-fold excess of unlabelled DHT (•). The binding pattern was analyzed at various running times. Only the SHBG peak is plotted in this figure.

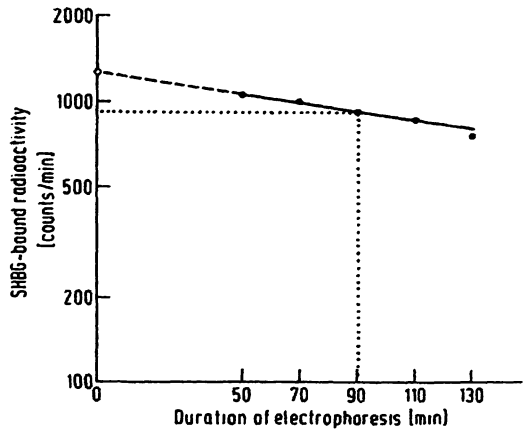

Fig. 6. Dissociation of the SHBG-bound [^{3}H]5α-dihydrotestosterone (DHT) during agar gel electrophoresis. The intercept of the extrapolated broken line with the ordinate indicates the SHBG-bound [^{3}H]DHT at time 0 of the electrophoresis. The dotted lines indicate the measured amount of SHBG-bound [^{3}H]DHT at 90 minutes. Mean values of two experiments are shown with deviation from experimental values of < 2%.

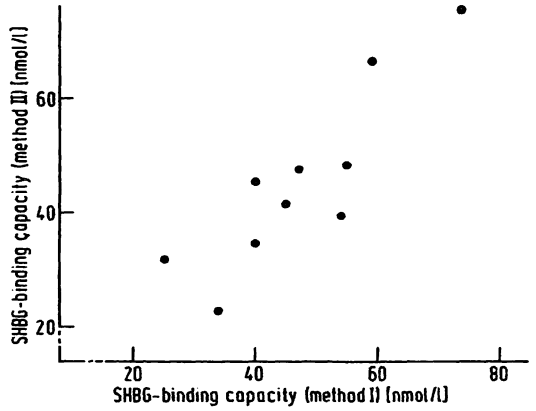

Fig. 7. *Spearman* rank correlation of SHBG-binding capacities of 10 male plasma samples, analyzed independently by two methods. Method I: agar gel electrophoresis; Method II: technique including ammonium sulphate precipitation and differential dissociation of the SHBG-[^{3}H]5α-dihydrotestosterone complex. n = 10, r = 0.83, p < 0.01.

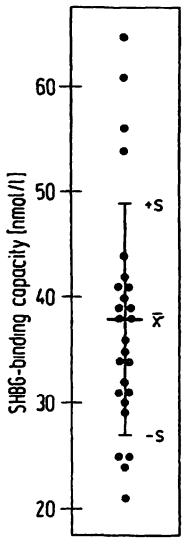

Fig. 8. SHBG-binding capacities in plasma of 26 normal males aged 22–41. The mean is indicated by $\bar{x}$; s = standard deviation.

Tab. 1. SHBG-binding capacities found by various authors in male plasma.

	$\bar{x}$ (nmol/l)	s	n	age
Wagner & Rüffert (12)	75	15	35	15–54
Shanbhag et al.*(8)	61	–	–	–
Ritzen et al. (6)	59	15	5	–
Rudd et al. (24)	50	4	10	21–37
Vermeulen et al. (16)	46	4	12	25–55
*Mickelson & Petra**(7)	44	0.3**	–	–
*Heyns & DeMoor**(17)	38	13	24	18–50
Krieg & Arning (this work)	38	11	26	22–41
Anderson et al. (18)	36	8	18	19–34
Rosner (19)	32	2**	–	–
Dennis et al. (15)	29	10	13	22–44
Corvol et al. (5)	17	1**	10	–

$\bar{x}$ = mean; s = standard deviation; n = number
* = calculated from μg/100 ml 5α-dihydrotestosterone binding
** = standard error of the mean

is obtained. We diluted the male plasma 1 : 21 and incubated it with 5.2 nmol/l [^{3}H]DHT. This concentration of [^{3}H]DHT is about three times higher than the average SHBG-binding capacity in undiluted male plasma. If extremely high SHBG concentrations are expected, e.g. in pregnancy plasma (12, 16), a ten-fold higher [^{3}H]DHT concentration was used to fulfill saturation conditions in a relatively wide range. *Wagner & Rüffert* (12) favoured a plasma dilution of 1 : 50 which gives, in our hands, relatively low binding peaks for male plasma (fig. 2), thus raising the statistical error of the counting rate of radioactivity from 2% to greater 5%.

Pretreatment of the plasma with charcoal, in order to remove endogenous steroids, which may occupy SHBG-binding sites, does not raise the binding capacity for [^{3}H]DHT. This is in accordance with findings reported elsewhere (6, 19, 20). Probably, within the 14 hour incubation period, the high excess of [^{3}H]DHT almost completely displaces endogenous androgens from the SHBG. Furthermore, charcoal might eliminate small amounts of SHBG itself from the incubation medium (13), thus masking the possible rise of the binding capacity. However, as shown by *Dennis* et al. (15), the loss of SHBG by charcoal adsorption is extremely low.

In contrast to the procedure of *Wagner & Rüffert* (12), we have introduced a competition step for quantifying the SHBG-binding capacity. As shown in figure 1, a

relatively high background was found in slice numbers 15–18 when incubating plasma with [^{3}H]DHT plus unlabelled DHT. If this background is not subtracted the actual SHBG-binding capacity would rise artificially. It should be noted that the displacement curve of figure 1 often displays a shoulder in slice numbers 15–18, indicating that small amounts of [^{3}H]DHT remain bound to SHBG. However, for routine determination of the binding capacity, this amount (less than 5% of the total SHBG) can be neglected.

Before this method was used routinely, the dissociation rate of [^{3}H]DHT from the SHBG-binding peak during electrophoresis was determined. The rate of 19% per hour is in good agreement with 16.5% found by *Wagner & Rüffert* (12). It may be anticipated that this rate is relatively constant, as it will be influenced only by monitored experimental conditions, e.g. electric field etc., but not by the individual plasma. Therefore we took the dissociation rate of 19% per hour as a constant factor for calculating the [^{3}H]DHT actually bound to SHBG.

The precision of the method is acceptable. The validity has been shown by the significant correlation of the data with the values obtained according to *Dennis* et al. (15), as well as by the comparison of normal ranges of SHBG-binding capacities in male plasma (table 1). The values of *Wagner & Rüffert* (12), also using agargel electrophoresis, are remarkably high. The main reason for this difference from our values might be that background subtraction has not been performed by these authors. The SHBG-binding capacities found in pregnancy plasma are in the range reported by others (5, 7, 8, 16, 17, 19).

The increase of the SHBG-binding capacity in elderly men has been described by various authors (15, 21, 22). Consequently, the percentage binding of testosterone to SHBG (23) will increase, amplified by the slightly lower testosterone plasma levels in elderly men (22). The biological implication of these changes lies in the assumption that only unbound plasmatic testosterone is biologically relevant.

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